Research Article

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Optimized purification of mono-PEGylated lysozyme by heparin affinity chromatography using response surface methodology

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Abstract

BACKGROUND: The efficient, controlled and robust purification of conjugates from PEGylation has a growing demand in the biopharmaceutical's market. In general, the yield and purity reached through the conventional chromatographic modes are not particularly high or efficient. Affinity chromatography has so far scarcely been explored. The present work introduces the purification of mono-PEGylated lysozyme from a PEGylation reaction by heparin affinity chromatography (HAC) for the first time in a single step. Response surface methodology (RSM), particularly a Box–Behnken design (BBD) was employed to optimize the separation.

RESULTS: Protein adsorption of PEGylated and native lysozyme on Heparin Sepharose 6 Fast Flow resin was described by Langmuir isotherms, showing a relatively low affinity for the PEGylated proteins. From the experimental design, optimal elution conditions in a linear gradient of sodium chloride (NaCl) for the three response variables (yield, purity and productivity) were: gradient length of 13 column volumes (CVs), flow at 0.8 mL min^{-1} and protein load of 1 mg mL^{-1}. Based on this optimization, a step gradient procedure was designed that achieved the purification of mono-PEGylated lysozyme with approximately 100% yield and purity in comparison with 92.7% and 99.7% with the linear gradient. Productivity was *c.* 0.048 ± 0.001 mg mL^{-1} min^{-1} using 0.05 mol L^{-1} NaCl for its elution.

CONCLUSIONS: Mono-PEGylated lysozyme was completely separated from a PEGylation mixture with high yield and purity using HAC for first time. Applying response surface methodology (RSM), adequate conditions for more than one requirement were found as well as optimal conditions for a linear gradient of NaCl. Based on this optimization a step gradient procedure was designed that achieved the purification of mono-PEGylated lysozyme in one step with advantages respect to time, resolution, yield and purity compared with other chromatographic modes such as hydrophobic interaction chromatography (HIC) and cation exchange chromatography (CEX).

Supporting information may be found in the online version of this article.

Keywords: heparin affinity chromatography (HAC); mono-PEGylated lysozyme; PEGylation; optimization; response surface methodology (RSM); Box–Behnken design (BBD)

INTRODUCTION

PEGylation is a drug delivery strategy which consists of covalently linked PEG (polyethylene glycol) to therapeutic proteins with the aim to improve significantly their biodistribution through positive characteristics among which are increased solubility, thermal and mechanical stability, reduced renal clearance, less immunogenicity and resistance to protease degradation.[1–3] There are several approved PEGylated proteins in the market used in the treatment of diseases,[4] meanwhile, others are still in clinical trials.[5]

Nowadays, the purification of PEGylated proteins is a bottleneck in their production due to the fact that a mixture of bio-conjugates with different PEGylation grades are generated in this reaction,[6,7] even when a site-specific PEGylation method is used.[8] Chromatography, due to the high resolution and purification obtained,[9] continues to be the preferred technique for the separation of PEGylated proteins from the reaction mixture. Size exclusion chromatography (SEC), ion exchange chromatography (IEX), reverse phase chromatography (RPC) and hydrophobic interaction chromatography (HIC) have been the modes used, however, these present several drawbacks among which are the

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sample dilution, process times, low recovery and loss of biological activity[8,10–12] of the PEG-conjugate. The total separation in these modes is reached with more than one chromatographic step. Thus, only affinity chromatography (AC) appears a suitable option.[13–15]This is a selective, high-level purification, fast, single step, reversible and mild technique[14,16] used for separation of proteins, enzymes, antibodies, hormones, receptors, factors, vitamins, nucleic acids, cell components, viruses and phages.[14] Few publications refer to the combined use of PEGylation and AC. Some have modified the stationary phases with PEG and studied their impact in the chromatographic separation of antibodies and glucose oxidase[17,18] or the use of AC in on-column PEGylation[19] to protect the enzyme´s active site. Nevertheless, purification of PEGylated proteins through AC has not been widely studied and characterized.

Heparin affinity chromatography (HAC) contains heparin as affinity ligand, a negatively charged (sulphated) glycosaminoglycan (alternating hexuronic acids with glucosamine residues) capable of binding to a wide range of biomolecules.[20,21] Mainly, HAC has been used for the fractionation of proteins and coagulation factors in serum,[22] and also in the isolation of proteases, lipoproteins, polymerases, nucleic acids, growth factors in bovine bone marrow and proteins from diverse sources.[21,23,24] Heparin is a high-cost ligand since it is obtained from animal sources, however, recent advances in chemoenzymatic synthesis of heparin and other related oligosaccharides are encouraging its large-scale production.[25–27]

In addition, several process variables have to be taken into account during the operation design in a short time with limited availability of protein. In this point, the design of experiment strategies such as response surface methodology (RSM) plays an important role and their use can provide advantages such as fast, control, scalability, robustness and quality in product.[28,29] Despite the fact that RSM is now being used more routinely in the design of unit operations in biotechnological processes,[30] its employment in preparative chromatography optimization has still been rare.[31,32]

In this work, the separation of PEG-conjugates of lysozyme produced by N-terminal site-specific PEGylation with 20 kDa mPEG-propionaldehyde in HAC is studied. Lysozyme is a protein model to study the PEGylation and the purification of conjugates in small therapeutic proteins, moreover, lysozyme presents antibacterial activity[33] and it functions as preservative and antibiotic synergist.[34] Batch adsorption of PEGylated and native lysozyme were characterized through isotherms. Recovery of mono-PEGylated lysozyme was optimized in linear gradient elution via Box–Benkhen design (BBD) by response surface methodology (RSM) to make the separation efficient. Using this result a highly efficient step gradient method was designed. So an optimized affinity chromatographic method is proposed for the purification of mono-PEGylated proteins.

MATERIALS AND METHODS

Materials

Lysozyme from chicken egg white (cat. no. 10837059001), barium chloride dehydrate (cat. no. B0750-100G) and iodine solution (cat. no. 319007-100 mL) were purchased from Sigma-Aldrich (MO, USA). Methoxy-PEG-propionaldehyde (cat no. A3001-10) with a nominal molecular weight of 20 kDa came from Jen Kem Technologies (TX, USA). Sodium cyanoborhydride (cat. no. 1001911397) was purchased from Fluka (MO, USA). Tris buffer grade (cat no. TR-16514) was supplied by Winkler LTDA (Santiago, Chile). Sodium chloride (cat no. 106404) came from Merck Millipore (MA, USA). Also Coomassie Brilliant Blue G used in the Bradford reagent preparation was obtained from Sigma-Aldrich. Heparin Sepharose 6 Fast Flow (cat no. 17099801) was purchased from GE-Healthcare (Uppsala, Sweden). All solutions were made using Milli-Q-grade water (Merck Millipore, MA, USA).

Preparation of PEGylated lysozyme standards

The di- and mono-PEGylated lysozyme standards were obtained from the purification of lysozyme PEGylation reactions. PEGylation reactions were prepared as described by Daly *et al.* and Cisneros-Ruiz,[35,36] consisting of a solution of 5.5 mL of lysozyme at 3.0 mg mL^{-1} in 100 mmol L^{-1} sodium phosphate buffer pH 5.1 with 20 mmol L^{-1} sodium cyanoborhydride, and 82.5 mg of 20 kDa mPEG-propionaldehyde stirred for 17 h at 4 °C. The reaction mixture was resolved by size exclusion chromatography (SEC) on Äkta Explorer System (GE Healthcare, Uppsala, Sweden) with a Sephacryl S-300 Hi Prep column (2.6 cm ID, 60 cm long, GE Healthcare, Uppsala, Sweden) at 1 mL min^{-1} using 10 mmol L^{-1} sodium phosphate buffer pH 7.2, containing 150 mmol L^{-1} potassium chloride.[11] Fractions absorbing at 280 nm were collected and concentrated by ultrafiltration with a 10 kDa Diaflo membrane (Amicon Inc., MA, USA) in an Amicon chamber. Finally, proteins were lyophilized and stored at −20 °C.

Batch adsorption

The adsorption of native and PEGylated lysozyme on Heparin Sepharose adsorbent was carried out individually by batch experiments at room temperature at different concentrations. Total volume of gel slurry was equilibrated for 0.5 h with 5 volumes of 20 mmol L^{-1} Tris–HCl pH 7.5 (buffer A) and dispensed into 1.5 mL microcentrifuge tubes (0.12 mL of gel slurry per tube). Then the resin was covered with 5 volumes of binding solution adding a mixture of buffering solution and protein solution to obtain final concentrations in the range of 0.5 mg mL^{-1} to 4 mg mL^{-1} of protein. Adsorption equilibrium was measured after incubation in a thermomixer comfort (Eppendorf, NY, USA) for 5 h at 1000 rpm. After that, the solution was removed by centrifugation at 9800 rpm for 5 min, the resin was washed with buffer A and protein desorption was done with 20 mmol L^{-1} Tris–HCl pH 7.5 containing 2 mol L^{-1} NaCl. All experiments were carried out in triplicate. Adsorbed protein (q_e) was calculated by a mass balance from protein determination in solution using the Bradford assay[37] in a spectrophotometer. Prior to sample analysis, each protein was calibrated in both buffers. Equilibrium adsorption capacity, q_e (mg g^{-1}), was calculated by mass balance as shown in Equation (1), considering Co and Ce, the initial and equilibrium protein concentrations (mg mL^{-1}), V, the volume of aqueous solution (mL) and m, the heparin adsorbent mass (g).

$$q_e = \frac{((Co - Ce) * V)}{m} \tag{1}$$

Three adsorption models (Langmuir, Freundlich and Temkin) commonly described for protein adsorption were tested. The respective equations are the following:[38]

$$q_e = \frac{q_o\, bC_e}{1 + bC_e} \tag{2}$$

$$q_e = K_F Ce^{\frac{1}{n}} \tag{3}$$

$$q_e = \frac{RT}{B_t} ln\,(At * Ce) \quad (4)$$

where q_o is the maximum monolayer adsorption capacity (g L_{gel}^{-1}) and b is the Langmuir isotherm constant in the Langmuir model (L g^{-1}). K_F is the Freundlich isotherm constant ((g L_{gel}^{-1})*(L g^{-1})$^{2.36}$) and n is the adsorption intensity. In the Temkin equation, R is the Universal Gas Constant (8.314 J K^{-1} mol^{-1}), T is temperature (K) and B_t is the Temkin isotherm constant and At is the Temkin isotherm binding equilibrium constant (L g^{-1}).

PEG test binding

In order to study the non-specific interactions between PEG and the heparin adsorbent, 0.12 mL of gel slurry were put in contact with PEG solution at 3 mg mL^{-1} as described for protein batch adsorption. Binding, washing and elution solutions were analyzed for PEG quantification by the iodine/barium chloride method as reported by Gong *et al.*,[39] and the amount of PEG adsorbed was calculated by mass balance as for protein.

Chromatographic method

Chromatographic experiments were performed in an Äkta Purifier 10 System (GE Healthcare, Uppsala, Sweden) equipped with a 200 μL injection loop. Heparin Sepharose 6 Fast Flow was flow packed in a 1 mL HR 5/5 column (100 mm × 5 mm ID). Packing performance was checked injecting 1% acetone pulses. All experiments were performed at room temperature. The liquid phases used in the chromatography were 20 mmol L^{-1} Tris–HCl pH 7.0 (buffer A) and 20 mmol L^{-1} Tris–HCl pH 7.0 containing 1 mol L^{-1} sodium chloride (buffer B). Solutions were filtered on a membrane of 0.2 µm (Advantec, WI, USA) and subsequently degassed. The column outlet was monitored at 215 nm. Two elution modes were tested: an increasing linear salt gradient and a step salt gradient, which are described below. The yield and purity were calculated using the plate model according to Belter.[40] Productivity was estimated as before.[40,41]

Linear gradient

Some test runs were done injecting protein mixtures of mono-PEGylated and native lysozyme in buffer A as a representative solution of the lysozyme PEGylation reaction. The separation was done with a linear salt gradient from A to 100% B. As mono-PEGylated and native lysozyme were not totally separated, optimization was suggested by design of experiments. For the experimental design the linear gradient elution mode was done using different conditions: gradient length (CVs), flow (mL min^{-1}) and protein load (mg mL^{-1}) according to the points generated in the experimental design. Once the separation conditions were optimized, the individual standards (di-PEGylated, mono-PEGylated and native lysozyme) were evaluated at those operational conditions to identify retention and elution behavior of the proteins.

Design of experiments (DoE) and result analysis in linear gradient

Response surface methodology (RSM) was applied to optimize the purification of mono-PEGylated lysozyme in HAC by linear gradient elution using as proof of concept a mixture of mono-PEGylated and native lysozyme at a ratio 4:1; this proportion was chosen based on previous knowledge about the concentrations at the end of the PEGylation reaction of these proteins. Di-PEGylated lysozyme was omitted in this mixture because it was not retained by the chromatographic column. The effect of gradient length from 5 to 25 column volumes (CVs), flow from 0.8 to 1.2 mL min^{-1}, and protein load from 0.25 to 1.75 mg mL^{-1} in three response variables: yield (%), purity (%) and productivity (mg mL^{-1} min^{-1}) were evaluated. A Box–Behnken design (BBD) with two central points and two replicates was generated on Minitab software and the experiments were done according to the specified run order. The data was analyzed individually for each response variable on the same software. Non-significant terms in the quadratic model were eliminated until a reduced model was obtained. The model fit was checked by analyzing lack of fit and the R^2. Also, the analysis of variance (ANOVA) assumptions were verified: normality, constant variance and independence of the residuals. The three response variables were jointly maximized through the 'optimizer' option on Minitab. Target value, minimum and importance values were defined according to the current requirements and end-use of mono-PEGylated lysozyme and these are indicated in Table A1. Optimal conditions were slightly adjusted for practical operational purposes.

The surface plots were created for the different responses studied. To confirm the optimum predicted average from the model the number of replicates was obtained with 'power and sample size' from Minitab. After performing the appropriate replicates at optimal conditions comparison with predicted responses was done using a one sample t-test at 95% confidence level. Finally, confidence intervals were computed for the optimum predicted average.

Step gradient

In order to improve the purification of mono-PEGylated lysozyme, a step gradient was developed with a lysozyme PEGylation reaction diluted 1:3 in buffer A at a flow of 0.8 mL min^{-1}. The initial concentrations were established according to the corresponding percentages of phase B where the mono-PEGylated and native lysozyme were eluted in the linear gradient; the method was modified to achieve the separation. The step gradient started with 5% phase B (5 CVs), 25% phase B (5 CVs) and 100% phase B (2 CVs).

RESULTS AND DISCUSSION

Adsorption isotherm

Adsorption data of proteins were tested with the linearized equations of three adsorption models: Langmuir, Freundlich and Temkin models which are the most frequently used models in the literature to describe protein adsorption on chromatographic resins[42–44] including affinity chromatography,[14,45] the regression coefficient (R^2) was the criteria for the selection of the model to fit. For the three proteins (di-, mono-PEGylated and native lysozyme) the best adjustment was observed with the Langmuir model ($R^2 = 0.997$, $R^2 = 0.908$, $R^2 = 0.977$, respectively) (Fig. S1), while in the other models the values of regression were low (in the Freundlich model these were: 0.635, 0.841 and 0.932 and in the Temkin model these were 0.960, 0.902 and 0.643, respectively). The Langmuir behavior has been observed in most of adsorption studies with PEGylated proteins on other chromatographic supports, mainly on ion exchange or hydrophobic adsorbents.[46,47]

Of the three proteins studied in batch adsorption, mono-PEGylated lysozyme showed the highest adsorption capacity (Table 1) (10.8 g L^{-1} of gel) with the Langmuir model followed in decreasing order by native and di-PEGylated lysozyme

Table 1. Estimated parameters for Langmuir isotherms of native and PEGylated lysozyme

Langmuir parameter	Native lysozyme	Mono-PEGylated lysozyme	Di-PEGylated lysozyme
qo ($g\,g^{-1}$)	4.88	10.79	2.80
b ($L\,g^{-1}$)	41.38	2.97	5.20
Ka (M^{-1})	6.08×10^{-5}	1.03×10^{-5}	2.85×10^{-5}

with 4.9 and 2.8 g L^{-1} of gel, respectively. It was expected that the mono-PEGylated conjugate had less binding capacity compared with the native protein due to the change caused by PEG-modification which has also been observed with other PEGylated proteins[46,48] on ion exchangers, nevertheless the discrepancy may be explained because the adsorption at static conditions not only might be driven by the ionic interaction with the protein but also with the PEG chain slightly as has happened on hydrophobic resins.[49]

The low capacity observed for di-PEGylated lysozyme could be more related to reduced accessibility to the pore space of the resin due to the increased size of the protein. Regarding the Langmuir association constant or affinity constant, native lysozyme had the highest affinity ($6.08 \times 10^5\ M^{-1}$) and it was lower for mono-PEGylated ($1.03 \times 10^5\ M^{-1}$) and di-PEGylated ($2.85 \times 10^5\ M^{-1}$) lysozyme; this value shows that PEGylation decreased the affinity of lysozyme for the resin. Examples where PEGylation also influences the affinity of the proteins are the reduction in the binding for glucose oxidase of PEGylated concanavalin A[17], and the increase of the dissociation constant (kd) with anti-native RNase antibody when ribonuclease was modified with 4 and 9 mPEG 5000 molecules.[50]

The 20 kDa mPEG-propionaldehyde exhibited a binding percentage between 1.0 and 3.0%, which was not considered significant, hence it appears there are no interactions between the PEG and the heparin adsorbent.

Heparin affinity chromatography (HAC) purification

Initial chromatographic tests in the 1 mL packed column with Heparin Sepharose 6FF showed a separation profile for PEGylated and native lysozyme using a linear gradient of NaCl at 1 mol L^{-1} (Fig. 1). Di-PEGylated lysozyme was not retained in the column at dynamic conditions and it was eluted in the washing step. This result is different from the detected binding in the batch isotherms but the effect of the flow could have affected the retention of the di-PEGylated conjugate. The mono-PEGylated and native lysozyme appeared early and relatively close in the elution at low salt concentrations as can be seen in Fig. 1. The identity of the peaks separated from the reaction components was verified with the injection of the individual standards. The amount of salt required to elute unmodified lysozyme (0.2–0.35 mol L^{-1} NaCl) agrees with the reported 0.3 mol L^{-1} NaCl for lysozyme purification from egg white at pH 7.4 on heparin-Ultrogel A4R.[51] The observed elution order of lysozyme isomers in the present study correlates with that for PEGylated proteins on cation exchange supports,[48,52] however, the profile presented here for HAC shows the complete resolution between mono- and di-PEGylated lysozyme conjugates which was not achieved in cation exchange chromatography (CEX) using Toyopearl Gigacap S-650 M and TSKgel SP-5PW resins.[48,52] Based on this and the role of heparin as a weak cation exchanger with some proteins,[53] the affinity between lysozyme and heparin could be influenced mainly by an ionic effect, by decreasing of the charge in PEGylation caused by the PEG addition;[54] however, no experimental demonstration is shown and affinity may be affected by different kind of interactions such as: hydrophobic interactions, hydrogen bonding or Van der Waals forces.[55] More studies to determine the exact nature of the interactions between lysozyme and PEGylated proteins need to be done.

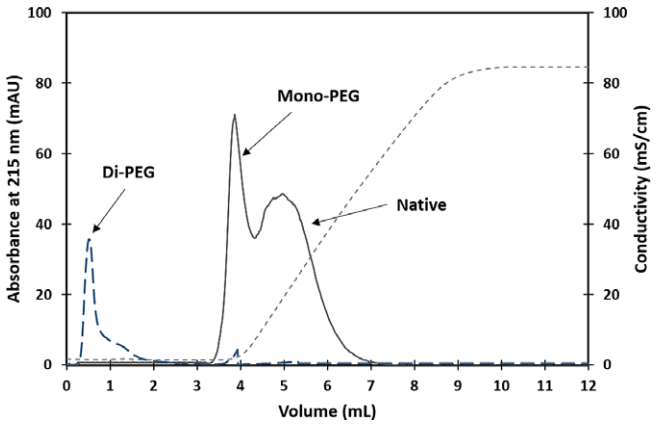

Figure 1. Chromatographic profile in initial tests for the separation of a lysozyme PEGylation reaction (1:3) separation on HAC using a protein mixture of di-PEGylated lysozyme, mono-PEGylated and native lysozyme. Buffer A: Tris–HCl pH 7.5. Buffer B: Tris–HCl pH 7.5 containing 1 mol L^{-1} NaCl; linear gradient from A to B of 5 CVs, flow rate: 0.8 mL min^{-1}, loop: 200 μL. [Di-PEGylated lysozyme]: 2 mg mL^{-1}, [Mono-PEGylated lysozyme]: 4 mg mL^{-1}, [Native lysozyme]: 1 mg mL^{-1}.

Optimization of linear gradient purification by response Surface methodology (RSM)

Since an incomplete separation of the mono-PEGylated and native lysozyme in the PEGylation reaction was observed, optimization of the separation of mono-PEGylated lysozyme in the linear elution gradient was pursued. In this optimization response surface methodology (RSM) was applied through a Box–Behnken design (BBD). Although RSM has already been applied in chromatographic purifications of several therapeutic products and proteins such as recombinant erythropoietin on Blue-Sepharose,[56] so far the application of RSM for the optimization of the chromatographic purification of PEGylated proteins has not been explored. The experimental points were done with a mixture of the above-mentioned proteins in a 4:1 ratio. The factor protein load and its levels were chosen based on the maximum amount of protein available to test this variable; gradient length and flow levels were studied in a reasonable space.

The analysis of variance (ANOVA) results for the yield, purity and productivity is shown in Table 2. In addition, Fig. 2 depicts the corresponding surface graphs. The lack of fit *P*-values in the three response variables: yield (0.537), purity (0.450) and productivity (0.254) were not significant, proving the fitting of the models. Also, the corresponding coefficients of determination (R^2) (yield (92.80%), purity (93.31%) and productivity (99.53%)) suggest the data and the variation in each response is explained by that percentage with the model. ANOVA assumptions (normality, constant variance and independence of the residuals) were verified and satisfied for all the models developed.

For yield, the protein load quadratic term was deleted from the model and the interaction flow-protein load was significant; protein loads from 0.25 mg mL^{-1} up to 1.0 mL min^{-1} combined

Table 2. ANOVA for the BBD design in the optimization of mono-PEGylated lysozyme separation in linear elution gradient of HAC. *P*-value < 0.05 was considered significant

Source	Sum of squares	Degrees of freedom	Mean square	F-value	*P*-value
Yield (%) ANOVA					
Model	1215.40	6	202.567	54.72	0.000
GL	504.03	1	504.032	136.16	0.000
F	211.46	1	211.457	57.12	0.000
PL	204.12	1	204.117	55.14	0.000
GL^2	196.17	1	196.173	52.99	0.000
F^2	51.82	1	51.824	14.00	0.001
F*PL	89.76	1	89.755	24.25	0.000
Error	70.33	19	3.702		
Lack of fit	20.29	6	3.382	0.88	0.537
Pure error	50.04	13	3.850		
Total	1285.74	25			
Purity (%) ANOVA					
Model	5.69509	6	0.94918	59.13	0.000
GL	4.31269	1	4.31269	268.65	0.000
F	0.42639	1	0.42639	26.56	0.000
PL	0.08728	1	0.08728	5.44	0.031
GL^2	0.73327	1	0.733327	45.68	0.000
PL^2	0.16130	1	0.16130	10.05	0.005
GL*F	0.11243	1	0.11243	7.00	0.016
Error	0.30501	19	0.01605		
Lack of fit	0.09810	6	0.01635	1.03	0.450
Pure error	0.20691	13	0.01592		
Total	6.00010	25			
Productivity (mg mL*min) ANOVA					
Model	0.003041	7	0.000434	759.97	0.000
GL	0.001977	1	0.001977	3458.81	0.000
F	0.000938	1	0.000938	1641.16	0.000
PL	0.000022	1	0.000022	38.26	0.000
GL^2	0.000032	1	0.000032	55.48	0.000
GL*F	0.000062	1	0.000062	108.64	0.000
GL*PL	0.000004	1	0.000004	7.1	0.016
F*PL	0.0000006	1	0.000006	10.37	0.005
Error	0.000010	18	0.000010		
Lack of fit	0.000004	5	0.000001	1.51	0.254
Pure error	0.000007	13	0.000001		
Total	0.003051	25			

GL = Gradient length, F = Flow, PL = Protein load.

with low flow (0.8 mL min^{-1}) (Fig. 2(A)) maximize the yield. With regards to purity, the flow quadratic term was omitted from the model since it was not significant, but the interaction gradient length–flow was held, keeping protein load at 1 mg mL^{-1}. Low flows and gradients between 15 and 25 CVs improve the purity (Fig. 2(B)), although in general the purities reached in the design are good. For productivity, all the interactions between the three factors were significant and the gradient length had an inverse impact on the productivity. Figure 2(C) shows the joint effect of gradient length and flow, the shorter the gradient (5 CVs) and the flow, the greater the productivity (0.050 mg of mono-PEGylated lysozyme (mg mL^{-1} min^{-1}). Regarding the gradient length–protein load interaction, if the gradient is around 5 CVs, regardless of protein load, productivity was between 0.040 and 0.045 (mg mL^{-1} min^{-1}). The interaction of flow–protein load at a gradient length of 15 CVs (Fig. 2(D)) indicates that if the flow goes from 0.8 to 0.9 mL min^{-1}, productivity will be between 0.035 and 0.040 (mg mL^{-1} min^{-1}), and it is the highest value if the flow is moved inside the design space.

Expressions describing quadratic models are listed in Equations (S1) to (S3) in Supplementary material. Since the optimal conditions for the three response variables were slightly different and opposed regarding productivity and yield, a joint optimization was carried out using the optimizer application of Minitab. The yield, purity and productivity were maximized and their respective top and objective values and importance; therefore, productivity was ranked with the highest importance followed by purities greater than 99%. The optimal conditions given by the optimizer were gradient length of 13.5 CVs, flow of 0.8 mL min^{-1} and protein load of 0.95 mL min^{-1} with a desirability or global satisfaction of the committed solution of about 0.89. Final conditions were fixed as 13 CVs for gradient length, 0.8 mL min^{-1} for flow and 1 mg mL^{-1} for protein load. The desirability for the modified conditions was 0.87, not

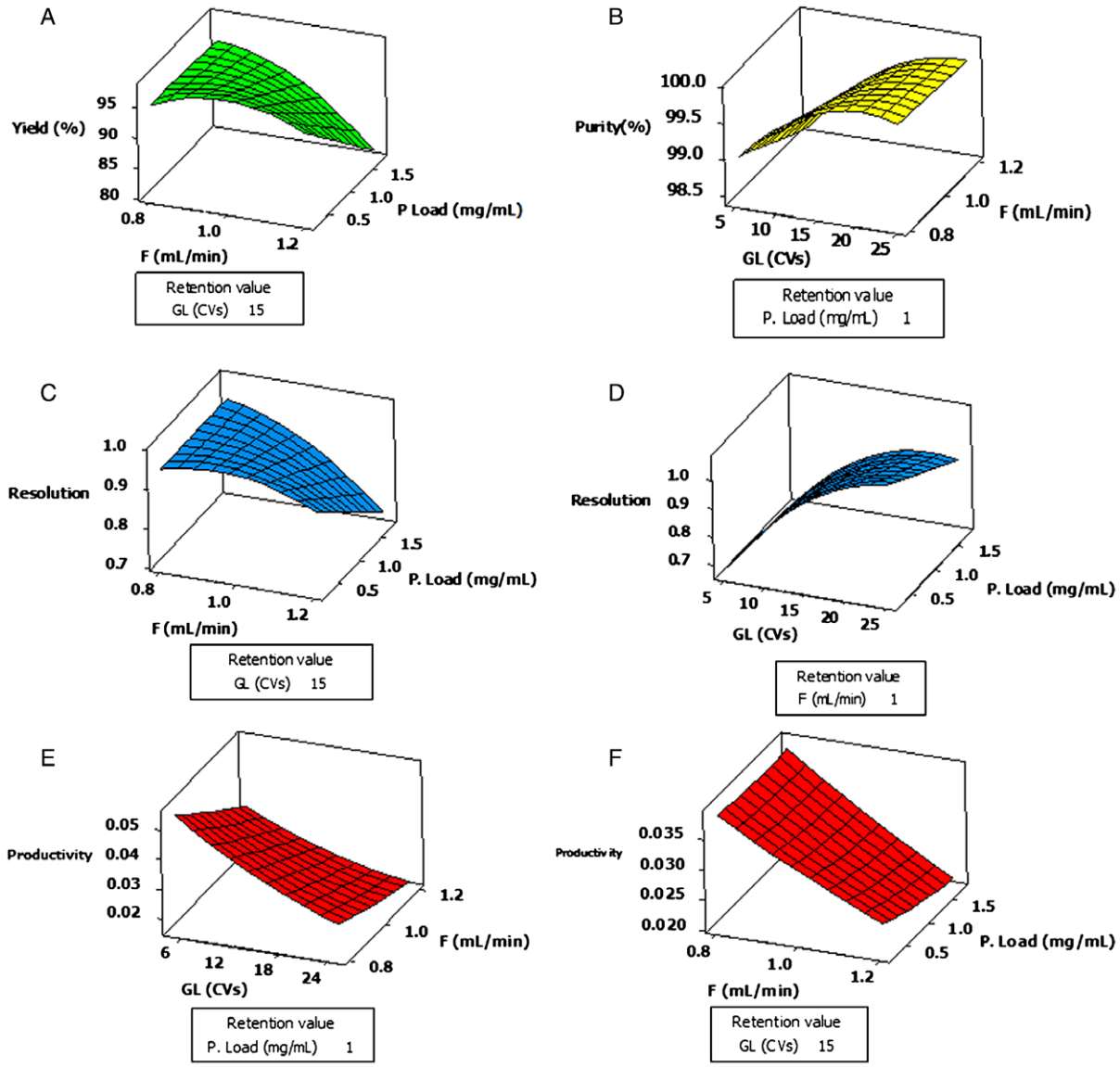

Figure 2. Surface plots for yield, purity, resolution and productivity responses. (A) Yield: flow versus protein load (gradient length fixed at 15CVs). (B) Purity: gradient length versus flow (protein load fixed at 1 mg mL^{-1}). (C) Resolution: flow versus protein load (gradient length fixed at 15CVs). (D) Resolution: gradient length versus protein load (flow fixed at 1 mL min^{-1}). (E) Productivity: gradient length versus flow (protein load fixed at 1 mg mL^{-1}). (F) Productivity: flow versus protein load (gradient length fixed at 15 CVs).

far from the previous estimation. These conditions make it possible to obtain mono-PEGylated lysozyme at a yield of 92.71%, purity of 99.69% and productivity of 0.0407 mg mL^{-1} min^{-1}. Finally, the model validation for each response variable was done running chromatographs at optimal conditions. In all the responses, the experimental values were not significantly different from the predicted values in the quadratic model (Table 3), confirming the precision of the model with a confidence level equal to or higher than 95% ($\alpha < 0.05$). All experimental response variables were in the range of the estimated confidence intervals (also included in Table 3). The chromatography results obtained by this optimization are shown in Fig. 3.

Step gradient purification

Based on the optimal conditions of the linear gradient and salt concentrations at which mono-PEGylated and native lysozyme eluted, 0.18 mol L^{-1} and 0.32 mol L^{-1} NaCl, respectively, a step gradient method was designed to separate the lysozyme PEGylation reaction products. Several tests were assayed changing the percentage of phase B and the duration of the step for the first two steps; the third was fixed at 100% of B. Almost the complete separation of proteins was achieved with a first step at 0.05 mol L^{-1} NaCl with 5 CVs, and a second step at 0.25 mol L^{-1} NaCl with 5 CVs (Fig. 4). These results point out that the small differences in the salt concentration make it possible to elute mono-PEGylated lysozyme from the native one (Fig. 4); the low NaCl concentration is favorable to recover the protein in solution and even the subsequent desalting operation may be omitted. One sign of total purification in the step gradient procedure is the resolution reached (2.35 ± 0.001), which is greater than 1.5, a resolution factor greater than or equal to 1.5 is considered a complete separation of peaks.[41] So, the yield and purity estimated by the plate model theory were 100% approximately; these values are 1.078 and 1.003 times greater than the yield and purity reached during the optimal conditions in the

Table 3. Statistic results in one-sample t test for quadratic model validation in the mono-PEGylated lysozyme HAC at optimal conditions using linear gradient (1 mol L^{-1} NaCl)

Response variable	Predicted value by model	Experimental value	Confidence level (%)	*P*-value	Estimated confidence interval
Yield (%)	92.71	93.76	95	0.182	91.52–95.99
Purity (%)	99.69	99.55	96	0.044	99.41–99.70
Productivity (mg mL^{-1} min^{-1})	0.0407	0.0406	95	0.634	0.0397–0.0415

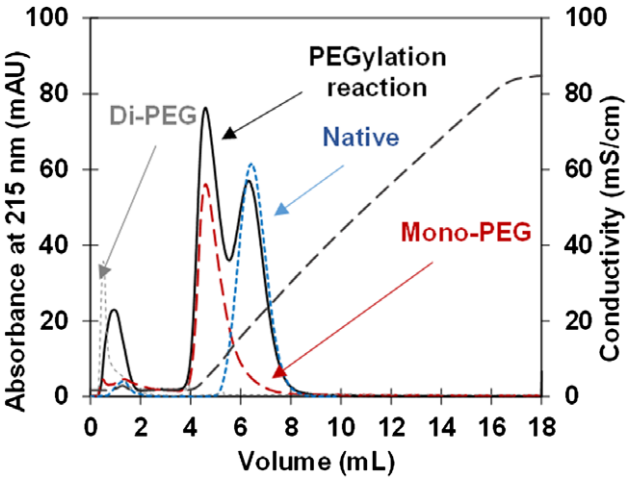

Figure 3. Chromatographic profile of purified di-PEGylated, mono-PEGylated, native lysozyme and a lysozyme PEGylation reaction (1:3) separation on optimized linear gradient conditions in HAC. Buffer A: Tris–HCl pH 7.5. Buffer B: Tris–HCl pH 7.5 containing 1 mol L^{-1} NaCl; linear gradient from A to B of 13 CVs, flow rate: 0.8 mL min^{-1}, protein load: 1 mg mL^{-1}, loop: 200 μL. [Di-PEGylated lysozyme]: 2 mg mL^{-1}, [Mono-PEGylated lysozyme]: 4 mg mL^{-1}, [Native lysozyme]: 1 mg mL^{-1}. Each protein standard and the reaction mixture were analyzed separately. The chromatograms were superimposed.

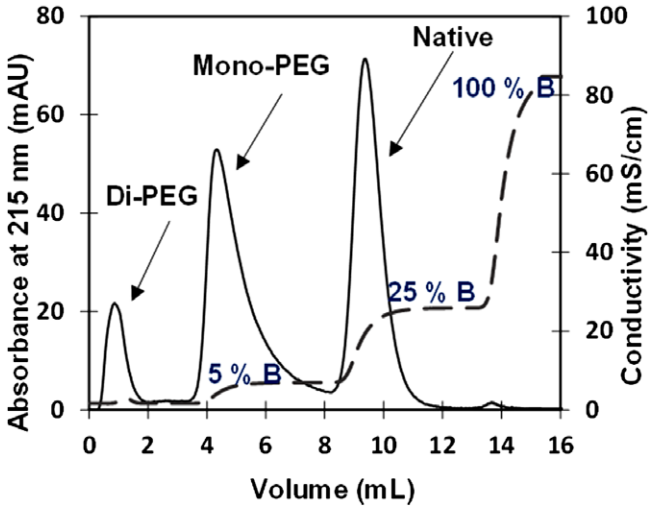

Figure 4. Step gradient elution profile of lysozyme PEGylation reaction (1:3) on HAC. Buffer A: Tris–HCl pH 7.5. Buffer B: Tris–HCl pH 7.5 containing 1 mol ,L^{-1} NaCl; step gradient from A to B. First step: 5% of B (5 CVs), second step: 25% of B (5 CVs), third step: 100% of B (2 CVs), flow rate: 0.8 mL min^{-1}, loop: 200 μL.

linear gradient, respectively. Productivity (0.048 ± 0.001 mg mL^{-1} min^{-1}) improved slightly with respect to the linear gradient prediction (0.041 mg mL^{-1} min^{-1}). Clearly, the step gradient procedure improves separation of the lysozyme PEGylation reaction.

Since no detailed information on yield and purity have been published for other chromatographic separations of mono-PEGylated lysozyme it was not possible to compare our results with previously reported ones. Our yields and purities (either those obtained by the optimized linear gradient or step gradient) are better relative to those reached for mono-PEGylated ribonuclease A purification in hydrophobic interaction chromatography (HIC) using butyl sepharose (85% yield and 97% purity)[57] and sepharose 6B-PEG5000 (96% yield and 85% purity).[58] In these separations the plate model was also applied to estimate yield and purity, and ribonuclease A is a protein with similar size to lysozyme (13.6 kDa vs 14.3 kDa), so it is a good guide to compare. The calculated productivity in the above-mentioned HIC purifications was 0.0039 mg mL^{-1} min^{-1} and 0.0031 mg mL^{-1} min^{-1}, respectively; these values are lower than those presented in this work.

Comparatively, HAC presented better resolution between conjugates than that observed when cation exchange resins separated 5, 10 and 30 kDa PEGylated lysozyme reactions.[48,52] Also, the separation with Toyopearl Gigacap S-650 M was longer (400 min)[48] than the required time to perform a chromatographic run in HAC at the optimized step gradient method (16.8 min). In summary, HAC for PEGylated proteins purification is a promising technique.

CONCLUSIONS

The adsorption of di-PEGylated, mono-PEGylated and native lysozyme to heparin Sepharose 6 Fast Flow adsorbent is described by the monolayer Langmuir model. The PEGylated conjugates had less affinity for the heparin adsorbent than the native protein while 20 kDa mPEG-propionaldehyde did not display unspecific binding with the resin.

A robust, efficient and novel chromatographic method for the purification of mono-PEGylated lysozyme from a PEGylation reaction mixture was developed with heparin affinity chromatography (HAC). The linear salt gradient elution using 20 mmol L^{-1} Tris–HCl with 1 mol L^{-1} NaCl was optimized via a Box–Behnken design, for which the adequate conditions used in the separation were a gradient length of 13 CVs, flow at 0.8 mL min^{-1} and protein load of 1 mg mL^{-1}. In this elution mode the predicted values by the model for the yield, purity and productivity were validated experimentally with an error level lower than 5% ($\alpha < 0.05$). The linear salt gradient found helped in designing a step gradient procedure to obtain a higher yield and purity of around 100%, approximately, and a productivity of 0.048 mg mL^{-1} min^{-1}. These yields, purities and productivities achieved for mono-PEGylated lysozyme by HAC are superior to those found in the purification of PEGylated proteins using other types of packed-bed chromatography, particularly HIC and advantageous in time saving and resolution with respect to CEX.

The optimization strategy implemented in the operation stage with response surface methodology (RSM) offers the possibility to streamline other chromatographic purifications with PEGylated

proteins as a first step to design proper, efficient and fast purification procedures.

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Supporting Information

Supporting information may be found in the online version of this article.

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